

Nicotine promotes *Streptococcus mutans* extracellular polysaccharide synthesis, cell aggregation and overall lactate dehydrogenase activity

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Footnote for abbreviations:

CLSM: Confocal laser scanning microscopy

EPS: Extracellular polysaccharides

Gtf: Glucosyltransferase

Gbp: Glucan binding protein

LDH: Lactate dehydrogenase

TSB: Tryptic soy broth

TSBS: Tryptic soy broth with 1% sucrose

Abstract

Several epidemiology studies have reported a positive relationship between smoking and dental caries. Nicotine, an alkaloid component of tobacco, has been demonstrated to stimulate biofilm formation and metabolic activity of *Streptococcus mutans*, one of the most important pathogens of dental caries. The first aim of the present study was to explore the possible mechanisms leading to increased biofilm by nicotine treatment from three aspects, extracellular polysaccharides (EPS) synthesis, glucosyltransferase (Gtf) synthesis and glucan-binding protein (Gbp) synthesis at the mRNA and protein levels. The second aim was to investigate how nicotine affects *S. mutans* virulence, particular in lactate dehydrogenase (LDH) activity. Confocal laser scanning microscopy results demonstrated that both biofilm bacterial cell numbers and EPS were increased by nicotine. Gtf and GbpA protein expression of *S. mutans* planktonic cells were upregulated while GbpB protein expression of biofilm cells were downregulated by nicotine. The mRNA expression trends of those genes were mostly consistent with results on protein level but not statistically significant, and *gtfD* and *gbpD* of biofilm cells were inhibited. Nicotine was not directly involved in *S. mutans* LDH activity. However, since it increases the total number of bacterial cells in biofilm, the overall LDH activity of *S. mutans* biofilm is increased. In conclusion, nicotine stimulates *S. mutans* planktonic cell Gtf and Gbp expression. This leads to more planktonic cells attaching to the dental biofilm. Increased cell numbers within biofilm results in higher overall LDH activity. This contributes to caries development in smokers.

Key words: *Streptococcus mutans*, nicotine, dental caries, glucosyltransferase, glucan binding protein, lactate dehydrogenase

Introduction

Dental caries, also known as tooth decay, is a chronic progressive disease demonstrated by tooth hard tissue loss caused by acid dissolution. Hydroxyapatite, which composes approximately 96% of tooth hard tissue, is soluble if the pH is less than 5.5 (1). *Streptococcus mutans* and Lactobacilli are the bacteria chiefly responsible for acid generation in caries lesions (2), and the presence of *S. mutans* is 70 times higher in caries-affected subjects than that in caries-free subjects (3).

Smoking is one of the leading causes of human death. Along with lung cancer, vascular stenosis, heart attacks and periodontal disease, dental caries has been reported to be associated with smoking all over the world, including Swedish and US smokers, Mexican truck drivers, Turkey and Japanese children, and Italy military personnel (4-9). A recent study in Japan demonstrated smokers received more caries/endodontic treatment than non-smokers (10).

Nicotine, an alkaloid accounting for 0.6-3% of tobacco, has been reported to promote *S. mutans* biofilm formation and biofilm cell metabolism (11). The nicotine concentration in saliva varies from 0 to 2.27 mg/ml in different subjects (12-15), and heavy smokers usually have high nicotine concentrations in their saliva (14). The major components of bacterial biofilm are bacterial cells, polysaccharides, proteins, lipids, extracellular DNA (eDNA), lipoproteins, lipooligosaccharides (16). Bacterial cells and extracellular polysaccharides (EPS) are the major components of biofilm matrix by dry weight. *S. mutans* EPS is synthesized by glucosyltransferase (Gtf) (17). There are three antigen groups related to *S. mutans* adhesion, antigen I/II, Gtfs and glucan-binding protein (Gbp). Antigen I/II is chiefly related to sucrose-independent binding. Gtfs and Gbps are chiefly related to sucrose-dependent binding, a major virulence factor of *S. mutans*. Another major *S. mutans* virulence factor is its ability to generate

lactic acid, which is regulated by *S. mutans* lactate dehydrogenase (LDH). LDH and lactic acid facilitates *S. mutans* to dissolve tooth minerals and cause dental caries. The aims of the present study were to investigate how nicotine affects *S. mutans* bacterial cell number and EPS synthesis within biofilm, and whether nicotine contributes to *S. mutans* LDH activity.

Materials and Methods

Chemicals, Bacterial Strains and Growth Conditions

(-)-Nicotine ($\geq 99\%$ (GC), liquid) was purchased from Sigma-Aldrich (St Louis, MO, USA). Nicotine, instead of smoking extract, was used because its chemical characteristics are specified. A single colony of *S. mutans* strain UA159 (ATCC 700610) was inoculated in tryptic soy broth (TSB) overnight before use. For each treatment, 1.0×10^6 CFU/ml *S. mutans* was used. Unless otherwise stated, the incubation atmosphere was 95% air/5% CO₂ at 37°C.

Confocal Laser Scanning Microscopy (CLSM)

S. mutans was grown in TSB plus 1% sucrose (TSBS) with 0, 1, 2 and 4 mg/ml nicotine and 1 μ M Alexa Fluor 647® red fluorescent dye (Dextran conjugates, 10,000 MW, Molecular Probes Inc., OR, USA) in four-well Lab-Tek Chamber slides (Thermo Fisher Scientific, Rochester, NY, USA) for 24 hours (18). The dextran conjugates were incorporated into EPS during biofilm matrix synthesis, and therefore it represented the amount of EPS. Biofilm was washed three times with diH₂O and incubated at room temperature for 10 minutes with 1 μ M SYTO® 9 green fluorescent dye (Nucleic acid stains, Molecular Probes Inc., OR, USA), which bound to bacterial nucleic acid and represented bacterial cell numbers in biofilm. Biofilm was then washed three times with diH₂O. After biofilm was air dried, ProLong Gold Antifade

Reagents (Molecular Probes Inc., OR, USA) was added to the biofilm to enhance its resistance to photobleaching. Fluorescent images were obtained by an Olympus FV1000-MPE Confocal/Multiphoton microscope (Olympus Corp., USA) with Olympus FV10-ASW software (Olympus Corp., USA) in the Division of Nephrology, Indiana Center for Biological Microscopy, Indiana University School of Medicine. The Alexa Fluor 488 and Alexa Fluor 647 fluorescent channels were selected to detect green and red fluorescence, respectively. The vertical distance between the highest and lowest scanning layers where biofilm presented was defined as biofilm thickness.

The image processing and analysis program was written according to Heydorn's principles (19) by MATLAB[®] R2012a (The MathWorks, Inc.) via the codes reported before (20).

Western Blotting

Western blotting was used to investigate *S. mutans* Gtfs, GbpA and GbpB protein expression. Those primary antibodies were kindly provided by Dr. Daniel J. Smith (Forsyth Institute, USA), and dnaK was kindly provided by Dr. Robert A. Burne (University of Florida). *S. mutans* was grown in TSBS with 0, 1, 2 and 4 mg/ml nicotine for 12 hours. Both planktonic and biofilm cells were harvested separately, washed three times with saline (0.9% sodium chloride) and suspended in lysis buffer [9 M urea, 2% nonidet-P40 and 1% dithiothreitol (DTT)]. Samples were sonicated [Branson Sonifier 450 (Branson Ultrasonics Corp., CO, USA) with duty cycle 50% and output control equal to 7] on ice with 0.2 mm microglass beads (B. Braun Melsungen AG., Melsungen, Germany) for 5 minutes in each cycle and for three cycles in total (21). Cellular proteins were harvested by centrifugation at 2,000 g for 15 minutes. The RC PC Protein Assay (BIO-RAD, Bio-Rad Laboratories, Inc., CA, USA) was used to determine

protein concentrations. The samples were mixed with an equal volume of sample buffer [64 mM Tris-HCl, pH 6.8, 25% glycerol, 0.15 mM bromophenol blue, 69 mM sodium dodecyl sulfate (SDS)] and incubated at 95°C for 10 minutes. Three µg samples from each treatment were loaded onto Tris-HCl electrophoresis gels (Ready Gel Tris-HCl Gel, Bio-Rad Laboratories, Inc., CA, USA), electrophoresed at 200 mA for 75 minutes and transferred to a polyvinylidene difluoride (PVDF) membrane at 30 V overnight. The PVDF membrane was then blocked in PBS + 0.1% Tween 20 + 5% non-fat milk (PBS-TM) for 2 hours, incubated with primary antibody dissolved in PBS-TM at appropriate concentrations (Gtfs 1:750, GbpA 1:2000, GbpB 1:5000, dnaK 1:2000) for 2 hours, washed three times with PBS-TM, incubated with anti-rat horseradish peroxidase secondary antibody dissolved in PBS-TM (Gtfs 1:1000, GbpA 1:5000, GbpB 1:5000, dnaK 1:5000) for 1 hour, washed three times with PBS-TM, rinsed with PBS, incubated with enhanced chemiluminescence substrate (ECL, Pierce ECL Western Blotting Substrate, Thermo Scientific, IL, USA) for 3 minutes and exposed to classic blue autoradiography film (Molecular Technologies, MO, USA)(22). dnaK was served as internal control. The intensity of bands was analyzed by ImageJ software (Version 1.48, NIH).

Quantitative-PCR (q-PCR)

The q-PCR protocol described previously was followed (20). Briefly, *S. mutans* was grown in TSBS with 0 and 2 mg/ml nicotine for 12 hours. Both planktonic and biofilm cells were harvested separately and washed three times with saline. Approximately 6×10^7 cells were stabilized by RNeasy Protect™ Bacteria Reagent (QIAGEN, MD, USA). Each *S. mutans* cell sample was suspended in 100 µl of lysis buffer (30 mM Tris-HCl pH 8.0, 1 mM EDTA and 15 mg/ml lysozyme) with 10 µl of 10 KU/ml mutanolysin (Sigma-Aldrich, MO, USA) and 15 µl of

proteinase K (QIAGEN), incubated at 37°C with agitation for 90 minutes, and sonicated for 5 cycles of 10 seconds/cycle (52% amplitude, Sonic Dismembrator, Model 500, Fisher Scientific). The RNA was isolated and purified by RNeasy Mini Kit (QIAGEN). RNA concentration was determined by NanoDrop 2000 (Thermo Fisher Scientific Inc., USA). SuperScript III First-Strand Synthesis System (Invitrogen™, Life Technologies Corp., CA, USA) was used to synthesize cDNA from 10 µg RNA. Fast SYBR Green Master Mix (Applied Biosystems, Life Technologies Corp., CA, USA), *S. mutans* *gtfB*, *gtfC*, *gtfD*, *gbpA*, *gbpB*, *gbpC* and *gbpD* primers (0.375 µM, Table 1) and cDNA template (2 µl) was processed for q-PCR replication (ABI PRISM® 7000 Sequence Detection System, Applied Biosystems, Life Technologies Corp.). q-PCR data was generated and analyzed by 7000 System SDS software (Applied Biosystems, Life Technologies Corp.) with all default settings. The original purified RNA samples were loaded with all the primers and Fast SYBR Green Master Mix for q-PCR as control to exclude genomic DNA contamination. The gene expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method described by Livak (23).

LDH Assay

LDH assays were used to determine cellular LDH activity of *S. mutans* treated by nicotine. Briefly, *S. mutans* was treated with 0, 0.5, 1, 2 and 4 mg/ml nicotine in TSBS for 24 hours, 45 µl of resuspended biofilm *S. mutans* cells was mixed with 5 µl of LDH Assay Lysis Solution and incubated in a microtiter plate for 45 minutes. Fresh 100 µl of LDH Assay Mixture (LDH Assay Cofactor Preparation : LDH Assay Substrate : LDH Dye Solution = 1:1:1) was added to 50 µl of cell lysate and the microtiter plate was incubated in the dark at room temperature for 30 minutes. The reaction was terminated by the addition of 15 µl of 1N HCl. The

absorbance was read at OD_{490nm}. The background absorbance at OD_{690nm} was measured and subtracted from the primary measurement, according to the manufacturer's manual. When the biofilm for the LDH assay was prepared, another set of biofilms for the crystal violet assay was prepared as well. Based on previous data (11), nicotine should stimulate *S. mutans* biofilm formation. It was necessary to adjust the LDH data by the amount of formed biofilm in order to estimate a unit cell of LDH activity. The crystal violet assay was processed as described before (11).

Statistical Analysis

Each experiment was independently repeated at least three times. All the experiments, except q-PCR, One-way Analysis of Variance (ANOVA) and the Tukey's multiple-comparisons test were used for statistical analysis by SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Student t-test was used for q-PCR data analysis. The level of significance was set at $P < 0.05$.

Results

Confocal Laser Scanning Microscopy (CLSM)

Both *S. mutans* bacterial cell numbers and EPS synthesis were increased by nicotine treatment. Bacterial cells in the 0 nicotine controls were spread out with several small aggregates, while the cells in the nicotine treated samples were more aggregated and contained larger aggregates (Fig. 1). There were less blank areas, which are indicative of biofilm channels, in the nicotine-treated groups than the non-treated groups. These results are consistent with previous scanning electron microscopy (SEM) data (11). Bacterial cell and EPS volumes were significantly increased in the 2 and 4 mg/ml nicotine groups. Bacteria and EPS coverage

demonstrated a trend to be increased by nicotine treatment, but only bacterial cell coverage at 4 mg/ml was statistically significant. Biofilm thickness was significantly increased at 4 mg/ml (Table 2).

Western Blotting

The GbpA and GbpB expression of planktonic cells was 8.1 and 2.5 fold higher than that of biofilm cells (Fig. 2, panel A). The Gtfs of biofilm cells was difficult to detect loading 3 µg of sample and its data was not included. Planktonic cell GbpA expression was significantly increased up to 4.0 fold by nicotine, Gtfs expression was increased up to 2.2 fold but this was not statistically significant, and GbpB was unchanged (Fig. 2, panel B). Biofilm cell GbpA was unchanged while GbpB was decreased up to 75% by nicotine treatment (Fig. 2, panel C).

q-PCR

The mRNA level of planktonic cells *gtfC*, *gbpA* and *gbpB* was increased 1.2 to 2 fold by nicotine, but none of the augmentation was statistically significant due to the large standard deviations (Fig. 3). The mRNA level of biofilm cells *gtfB*, *gtfC*, *gtfD*, *gbpB* and *gbpD* was 0.46 to 0.88 fold significantly decreased by nicotine. No significant genomic DNA was detected in the original RNA samples.

LDH Assay

S. mutans biofilm overall LDH activity was significantly increased at 1, 2 and 4 mg/ml nicotine (Fig. 4). However, after adjustment by the biofilm volume, which was determined by crystal violet assay (data not shown), there was no difference among those groups. This indicates

nicotine does not directly affect LDH activity of *S. mutans*, but with the increased bacterial cell number by nicotine treatment, the overall biofilm LDH activity was increased.

Discussion

This is a follow up study of a previous report (11). In the previous study, we demonstrated nicotine stimulates *S. mutans* biofilm formation and metabolism. SEM results indicated there were more bacterial cells and biofilm matrix in nicotine treated groups than the control group. However, the quantitative number of bacterial cells and EPS were unknown, and the detailed mechanisms for increased biofilm formation was not explored. Therefore, the present study was designed to address those questions.

From the CLSM results, both bacterial cells and EPS were significantly increased by nicotine treatment. To better understand biofilm structure, two parameters were used to interpret the figures. The first parameter is volume, which is calculated by adding the color intensity of each pixel on the image. This represents total cell numbers. The second parameter is coverage, which is calculated by counting the presence of color at each pixel site. For the image analysis, we used Heydorn's principles (19). But since theirs were 3-dimensional images and ours are 2-dimensional images, we deleted the z-axis factor from our algorithm. For bacterial cells, both volume and coverage were increased in a nicotine dependent manner (Fig. 1 and Table 2). This demonstrates that as more bacterial cells attach to the biofilm they occupy more space. For EPS, unlike bacterial cells, only EPS volume was increased with nicotine treatment. The coverage data indicated an increased trend but this was not statistically significant. The ratio of EPS/bacteria was not changed in 1 or 2 mg/ml nicotine groups but significantly increased in the 4 mg/ml nicotine group, this indicates the increased EPS was due to the nicotine treatment. EPS is

essential for bacterial adhesion-cohesion, and it plays a critical role in creating low pH microenvironments and providing binding sites for bacterial attachment (24). EPS also serves as a local sugar reservoir that can be fermented to acids, which contributes to caries development (17, 24). Therefore, the increased EPS synthesis by nicotine may increase smokers' caries risk.

Gtfs are a family of enzymes that catalyze the transformation of glucosyl groups from one chemical component to another and contributes to *S. mutans* cariogenicity (25). *S. mutans* expresses three distinct Gtfs, GtfB, GtfC and GtfD. They are encoded by *gtfB*, *gtfC* and *gtfD*, respectively. GtfB and GtfC are encoded by highly homologous genes and they primarily synthesize α -1,3-rich water-insoluble polysaccharide (26, 27), while GtfD is responsible for the synthesis of α -1,6-rich water-soluble polysaccharides (28). Although the roles of Gtfs are different from each other, their functions actually overlap as the water-soluble polysaccharide synthesized by GtfD is the primer for GtfB (29). The Gtfs (GtfB and GtfC) protein expression was very low in biofilm cells such that it could hardly be detected when similar amounts of biofilm cell total protein were loaded with planktonic cell total protein (data not shown). Planktonic cell Gtfs proteins were significantly increased at 2 and 4 mg/ml nicotine (Fig. 2, Panel C). In particular, the GtfC band (lower band in Western blot results) indicated more significant changes than the GtfB band (upper band). This is consistent with *gtfB* and *gtfC* gene expression (Fig. 3). The *gtfD* expression of planktonic cells was also demonstrated by an upregulation trend with nicotine treatment, but none of the *gtfs* upregulation was significant due to the large standard deviations. Typically, the RNA extraction from planktonic cells is easier than that from biofilm cells (30). But with the presence of sucrose, planktonic cell RNA extraction becomes more difficult than biofilm cell RNA extraction. Several solutions were tried, such as increasing lysing time, increasing sonication time, adjusting cell numbers, or extraction

with chloroform, but none of them worked. It was interesting that nicotine stimulated planktonic cell *gtfs* but inhibited biofilm cell *gtfs* (Fig. 3). *S. mutans* biofilm cell protein synthesis has been reported to be different from planktonic cell protein synthesis, such as glycolytic enzymes related to acid formation were inhibited in biofilm cells (31). *gtfs* expression was 2-3 fold lower in non-nicotine treated biofilm cells than planktonic cells (data not shown). One possible explanation for this phenomenon is the regulation effect of the *S. mutans* quorum sensing (QS) system, which senses microbial cell numbers, environmental stresses, carbohydrate types and its quantity (32). In planktonic status, the cell density was not changed (11), therefore the QS system does not work and the net stimulation effect of nicotine on *gtfs* is observed. But in biofilm status, increased cell density leads to QS system regulation that turned on the *gtfs* downregulation pathway to limit more biofilm cell accumulation. This decrease caused by the QS system offset the effect of nicotine on *gtfs*.

Theoretically, Gtfs belong to the Gbps family because they are related to cell binding. But in most literature, Gbps are defined as non-Gtf glucan-binding proteins that serve as cell-surface receptors for glucan (33). GbpA and GbpD share homology of glucan-binding domains with Gtfs. The *gbpA* knockout mutant strain forms a smoother biofilm than the wild type (34). That means *gbpA* makes biofilm coarse. Planktonic GbpA was increased four fold by nicotine (Fig. 2, panel C) and the *gbpA* also demonstrated a nicotine upregulation trend (Fig. 3). Those upregulations were correlated to the more aggregated and coarse biofilm surface observed in the CLSM (Fig. 1) and SEM results (11). GbpB is involved in bacterial cell-wall synthesis (35). The GbpB and *gbpB* expression in biofilm cells were significantly decreased with nicotine treatment. This decrease might be caused by autoinducer-2 (AI-2), a signaling molecule of QS system, which has been speculated to regulate *gtfs* and *gbps* expression (33). Since there is increased EPS

in the biofilm with nicotine treatment (Fig. 1), AI-2 downregulates GbpB expression. GbpC, which is related to bacterial biofilm structure (33), is not significantly affected by nicotine. The function of GbpD is similar to Gtfs and GbpA serving as a glucan-binding enzyme (33), and the effect of nicotine on *gbpD* is similar to *gtfs* and *gbpA* as well (Fig. 3). GbpA and GbpB expression in planktonic cells were eight and three fold higher than that in biofilm cells, respectively (Fig. 2, panel A), because of the absence of QS system in planktonic cells. Unfortunately no GtfD, GbpC and GbpD protein data was available due to the lack of primary antibodies. Since the gene and protein regulation happens before the final phenotype is observed, 12 hour samples was used for the Western blot and q-PCR while 24 hours samples was used for other experiments.

LDH is the enzyme that catalyzes the conversion of pyruvate to lactate with concomitant interconversion of NADH and NAD⁺ and produces lactic acid as the final product (36). *S. mutans* produces LDH constitutively if the fructose-1,6-diphosphate is present in adequate amounts (37) as the catalytic activity of LDH requires the presence of fructose-1,6-diphosphate in a pH range of 5 to 6.2 (36). LDH deficiency is lethal to *S. mutans* due to accumulated toxic intermediate products such as pyruvate (38, 39). Since LDH activity is directly related to *S. mutans* lactic acid production, LDH is considered one of *S. mutans* major virulence factors. Although nicotine does not directly regulate *S. mutans* LDH activity (Fig. 4, panel B), nicotine increases total bacterial cells in the biofilm, the overall LDH activity is increased (Fig. 4, panel A). *S. mutans* biofilm lactate generation was significantly increased with 1 mg/ml nicotine treatment (unpublished data).

In conclusion, nicotine stimulates *S. mutans* planktonic cell Gtfs and Gbps expression. This leads to more planktonic cells attaching to dental biofilm. Increased cell numbers within

biofilm result in higher overall LDH activity. More lactic acid may generated and contribute to caries development in smokers.

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Table 1. Specific primers for q-PCR

Primers		Sequences
16S rRNA	F	5'-AGCGTTGTCCGGATTTATTG-3'
	R	5'-CTACGCATTTACCGCTACA-3'
<i>gtfB</i>	F	5'-TACACTTTCGGGTGGCTTGG-3'
	R	5'-AGAAGCTGTTTCCCCAACAGT-3'
<i>gtfC</i>	F	5'-AGCAGATTCAACTGACGACCG-3'
	R	5'-TCAGTAACAGTGGCGGTTGG-3'
<i>gtfD</i>	F	5'-TGCAAGCGACGGAAAACAAG-3'
	R	5'-GCCTGTCAGAGCTTCACCAT-3'
<i>gbpA</i>	F	5'-TCATCAGGCACAGAACCACC-3'
	R	5'-CAGTTGAGGCTCGTTTCCCT-3'
<i>gbpB</i>	F	5'-AGGGCAATGTACTTGGGGTG-3'
	R	5'-TTTGGCCACCTTGAACACCT-3'
<i>gbpC</i>	F	5'-TCTGGTTTTTCTGGCGGTGT-3'
	R	5'-GTCAATGCTGATGGAACGCC-3'
<i>gbpD</i>	F	5'-TTGACTCAGCAGCCTTTCGT-3'
	R	5'-CTTCTGGTTGATAGGCGGCA-3'

Table 2. Biofilm coverage, volume and thickness of *S. mutans* treated with nicotine

Nicotine (mg/ml)	Bacteria		EPS		Thickness (μm)
	Volume ($\mu\text{m}^2/\mu\text{m}$)	Coverage (%)	Volume ($\mu\text{m}^2/\mu\text{m}$)	Coverage (%)	
0	47.67 \pm 6.45	35.33 \pm 3.84	69.33 \pm 13.08	55.38 \pm 12.24	38.67 \pm 3.42
1	50.80 \pm 4.47	40.32 \pm 10.09	71.36 \pm 11.25	60.41 \pm 10.01	41.06 \pm 0.69
2	60.27 \pm 4.82*	45.87 \pm 3.57	99.13 \pm 16.25*	72.75 \pm 13.26	42.95 \pm 2.81
4	77.60 \pm 6.47**	69.27 \pm 4.28*	96.90 \pm 4.75*	73.63 \pm 1.68	52.41 \pm 4.01*

Asterisks indicate significant difference from the 0 nicotine control sample. *P<0.05, and **: P<0.01.

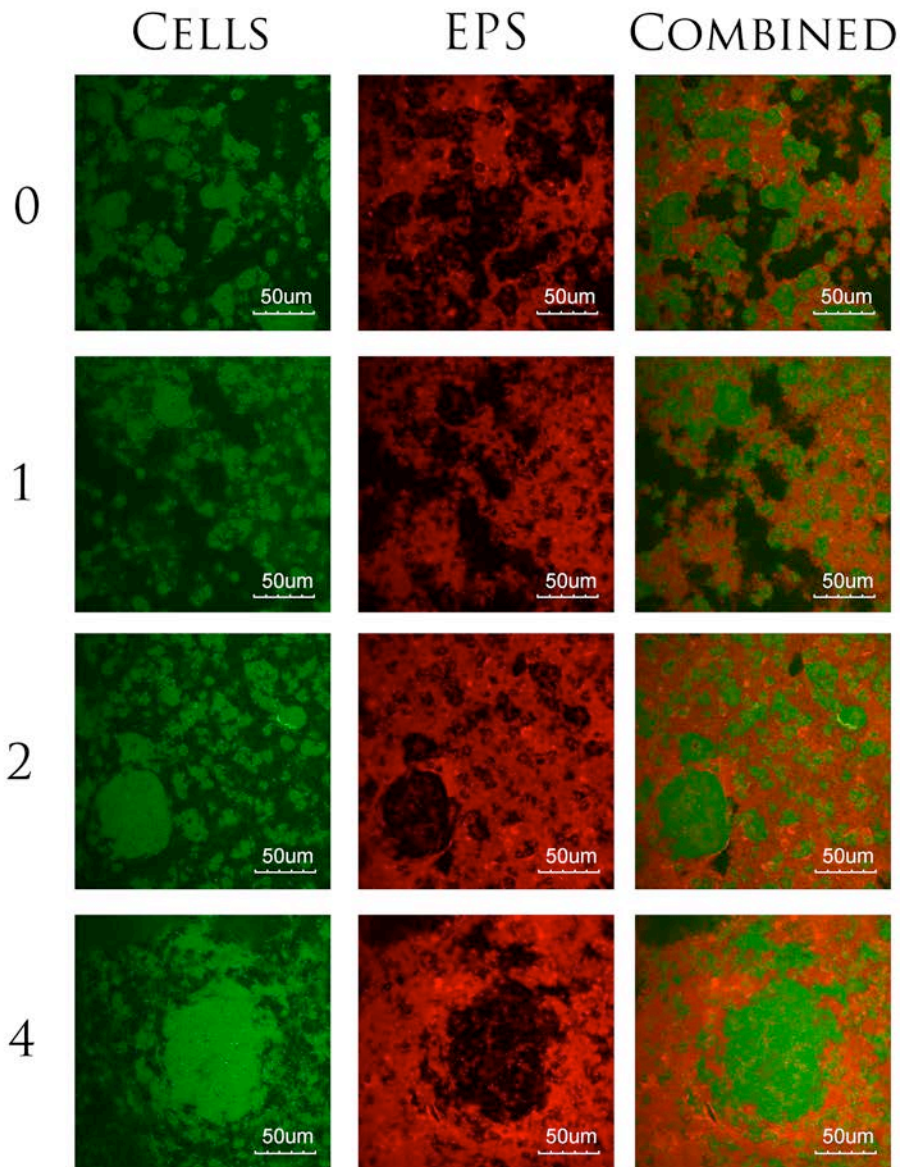
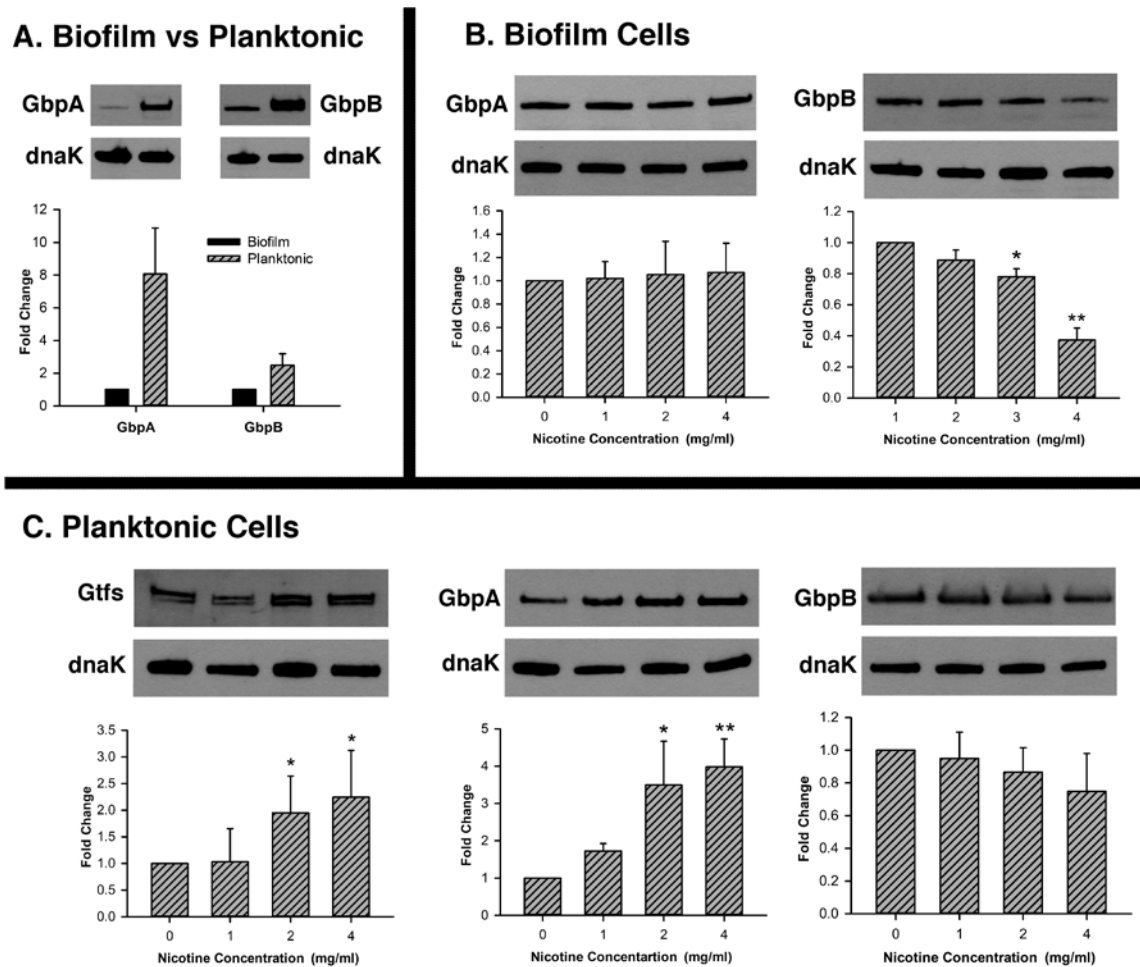


Fig. 1. Multiplication of *S. mutans* biofilm bacterial cells and EPS synthesis with nicotine.

S. mutans was treated with 0-4 mg/ml nicotine (left legend) for 24 hours in TSBS. Bacterial cells were labeled with SYTO® 9 green fluorescence and EPS was labeled with Alexa Fluor 647® red fluorescence. Results indicated bacterial cell multiplication and EPS synthesis were increased by

nicotine. Bacteria cells were more aggregated at higher nicotine concentrations than lower concentrations.

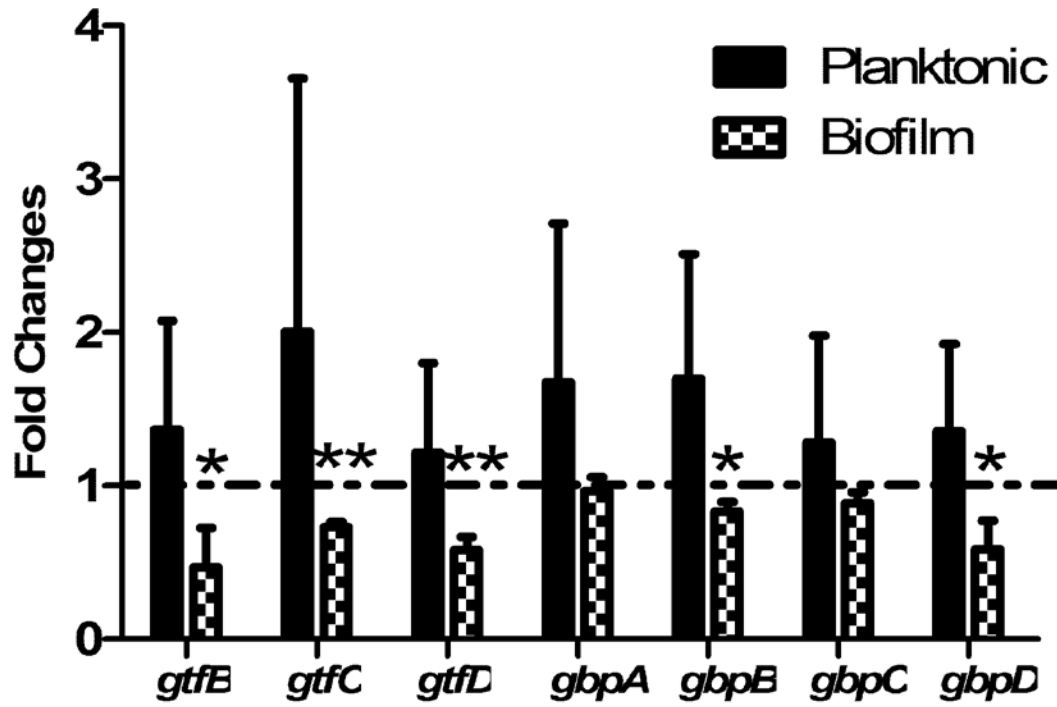


3 **Fig. 2. Western blot of *S. mutans* Gtfs and Gbps expression.**

4 *S. mutans* was treated with 0-4 mg/ml nicotine for 12 hours in TSBS. Both planktonic and
 5 biofilm cells were harvested. The expression of biofilm Gtfs could hardly be detected and its data
 6 is not shown. The band intensities of dnaK, which was used as internal control, were not shown
 7 in bar graphs. **Panel A** demonstrates the GbpA and GbpB expression of biofilm cells compared
 8 to planktonic cells. None of those samples were treated with nicotine. **Panel B** demonstrates the
 9 GbpA and GbpB expression of biofilm cells treated with different nicotine concentrations. **Panel**
 10 **C** demonstrates the Gtfs, GbpA and GbpB expression of planktonic cells treated with different

11 nicotine concentrations. Asterisks indicate significant differences from the 0 nicotine control
12 sample. * $P < 0.05$, and **: $P < 0.01$.
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16 **Fig. 3. *S. mutans* gtfs and gbps gene expression.**

17 *S. mutans* was treated with 0 and 2 mg/ml nicotine for 12 hours in TSBS. Both planktonic and
 18 biofilm cells were harvested for real-time PCR. The 2 mg/ml nicotine-treated q-PCR was shown.
 19 Black solid bars represent planktonic cell gene expression while gray streaked bars represent
 20 biofilm cell gene expression. The reference line of 1 indicated gene expression of the 0 nicotine
 21 group. Asterisks indicate significant differences between nicotine treated and non-treated
 22 samples. * $P < 0.05$, and **: $P < 0.01$.

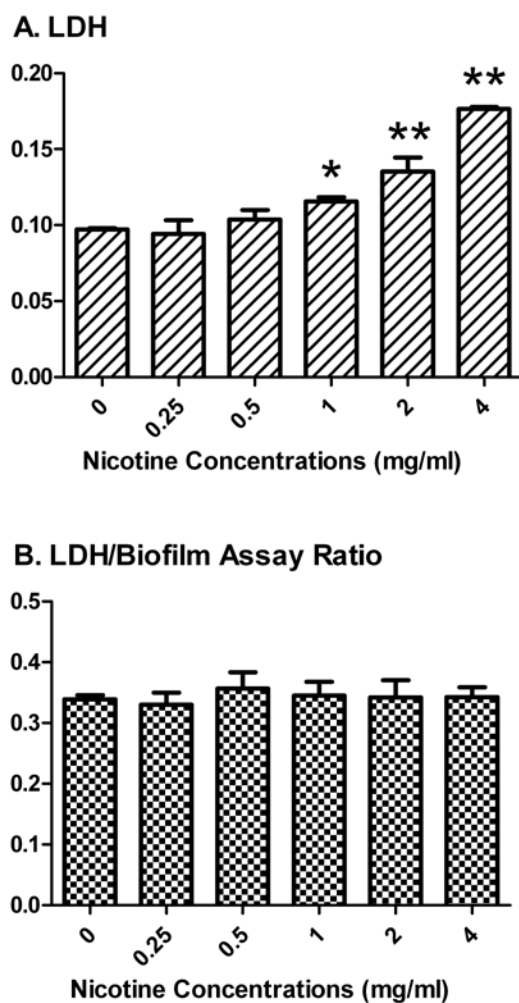
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30 **Fig. 4. LDH activity of *S. mutans* with nicotine.**

31 *S. mutans* was treated with 0-4 mg/ml nicotine for 24 hours in TSBS. Biofilm was washed and
 32 processed for LDH assay. The manufacturer's protocol was followed. **Panel A** demonstrates the
 33 overall biofilm LDH activity. Since nicotine stimulates *S. mutans* biofilm formation (Huang et al.,
 34 2012), crystal violet assay was used to estimate the amount of biofilm in each treatment (data not
 35 shown). **Panel B** demonstrates the ratio of LDH assay over the crystal violet assay data. This

36 represents unit cell LDH activity. Asterisks indicate significant difference between nicotine
37 treated and non-treated. * $P < 0.05$, and **: $P < 0.01$.